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The haem–copper oxygen reductase of *Desulfovibrio vulgaris* contains a dihaem cytochrome *c* in subunit II

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ABSTRACT

The genome of the sulphate reducing bacterium *Desulfovibrio vulgaris* Hildenborough, still considered a strict anaerobe, encodes two oxygen reductases of the *bd* and haem–copper types. The haem–copper oxygen reductase deduced amino acid sequence reveals that it is a Type A2 enzyme, which in its subunit II contains two *c*-type haem binding motifs. We have characterized the cytochrome *c* domain of subunit II and confirmed the binding of two haem groups, both with Met-His iron coordination. Hence, this enzyme constitutes the first example of a $ccaa_3$ haem–copper oxygen reductase. The expression of *D. vulgaris* haem–copper oxygen reductase was found to be independent of the electron donor and acceptor source and is not altered by stress factors such as oxygen exposure, nitrite, nitrate, and iron; therefore the haem–copper oxygen reductase seems to be constitutive. The KCN sensitive oxygen reduction by *D. vulgaris* membranes demonstrated in this work indicates the presence of an active haem–copper oxygen reductase. *D. vulgaris* membranes perform oxygen reduction when accepting electrons from the monohaem cytochrome c_{553}, thus revealing the first possible electron donor to the terminal oxygen reductase of *D. vulgaris*. The physiological implication of the presence of the oxygen reductase in this organism is discussed.

1. Introduction

Sulphate reducing bacteria of the *Desulfovibrio* genus are able to use a large diversity of inorganic compounds as electron acceptors [1]. Although considered for many years as strict anaerobes, several *Desulfovibrio* strains show high tolerance to oxygen [2–4]. This is particularly important, since sulphate reducers live in habitats close to the oxic/anoxic zones [5–7]. In particular, *D. desulfuricans* ATCC 27774 was recently reported to be able to grow in the presence of nearly atmospheric oxygen levels [4]. Several enzymes involved in the detoxification of reactive oxygen species (ROS) are proposed to sustain aerobic tolerance and to be involved in the reduction of oxygen to water [8]. For example, the cytoplasmic rubredoxin:oxygen oxidoreductase (ROO), one of the first examples of the family of flavodiiron enzymes [9,10], and the membrane-bound terminal oxygen reductase of the cytochrome *bd* family of *D. gigas* were shown to reduce oxygen to water [9,11]. Also, these organisms contain a plethora of systems that enable them to cope with reactive oxygen species, including superoxide dismutases and reductases, and catalases [8]. However, there is still no clear evidence in *Desulfovibrio* species for an oxygen reduction coupled to oxidative phosphorylation. Genes encoding a haem–copper oxygen reductase and a *bd*-type oxygen reductase are present in the genomes of *D. vulgaris* Hildenborough and *D. desulfuricans* G20[1] as well as a gene encoding a protohaem IX farnesyltransferase. The latter protein synthesizes haem *o* from the protohaem IX (haem *b*) which is further modified to yield haem *a*, the cofactor present in several haem–copper oxygen reductases.

Oxygen reductases are the last complexes in the membrane-bound respiratory chains of aerobic organisms, and can be divided into three major groups: the haem–copper oxygen reductases (also generally called cytochrome *c* oxidases), the *bd*-type oxygen reductases, which are solely quinol oxidases, and the alternative oxidases which contain a di-iron catalytic site and occur in plants, fungi, protists and in some bacteria [12–16]. The haem–copper oxygen reductases catalyze the four electron reduction of dioxygen to water and use the free energy released from the oxidation of periplasmatic metalloproteins by dioxygen to pump protons across the membrane [15–19]. Both the chemical reaction and the pumping of protons lead to the generation of a transmembrane difference of electrochemical potential, a key process in biological energy conversion.

The haem–copper oxygen reductases derive their name from the presence in the catalytic subunit (subunit I, the only subunit common to all members of this superfamily) of a centre constituted by a high-spin haem and a copper ion, where the reduction of molecular oxygen

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[1] It should be noted that *D. desulfuricans* G20 is in fact not related to the *Desulfuricans* strains, such as Essex and ATCC 27774.

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to water occurs; this subunit also contains a low-spin haem, the ultimate electron donor to the catalytic centre. The microbial enzymes have up to four subunits, whereas the eukaryotic enzymes are constituted up to thirteen subunits. The superfamily of haem–copper enzymes was divided into three families, A, B and C [19], according to amino acid sequence comparisons and to the conservation of amino acid residues proposed to be involved in the uptake and pumping of protons. This classification was later corroborated by the compilation and comparison of data acquired for the catalytic centre [20]. The A family, was further subdivided into subfamilies A1 and A2. The A1 subfamily comprises the mitochondrial and mitochondrial-like microbial enzymes that have several conserved amino acid residues (in the so-called D and K channels) which were shown by various studies to be important for proton uptake and pumping. The enzymes of the A2 subfamily contain all those residues, with the exception of a glutamyl present at the end of the D-channel, in helix VI: instead, they have a tyrosyl and a seryl in the same helix, one turn below, which were proposed to play a role in proton conduction [19]. Subunit II, conserved in A and B type enzymes, has either a binuclear average valence copper centre if it receives electrons from periplasm facing metalloproteins or no prosthetic group in the case of quinol oxidases. In some cases, this subunit contains an extra C-terminal domain, which harbours a *c*-type haem [19].

In this article we show that the *D. vulgaris* haem–copper oxygen reductase is a member of the A2 type enzymes, that it is expressed under a variety of conditions, and demonstrate that subunit II contains two *c*-type haems. Furthermore, the lack of information on the nature of the electron donors to the *Desulfovibrio* haem–copper oxygen reductases led us to explore the possibility that, in *D. vulgaris*, the monohaem cytochrome c_{553} performs this role since this protein is encoded by the gene *cyf* located in the vicinity of the genes encoding the haem–copper oxygen reductase.

2. Materials and methods

2.1. Cloning and expression of the cytochrome c domain of the D. vulgaris Hildenborough haem–copper oxygen reductase

In *D. vulgaris* Hildenborough the haem–copper oxygen reductase subunit II is encoded by the gene DVU1812 [21]. A truncated form of this gene was constructed to allow production of a protein comprising only the C-terminal region that contains binding motifs for two haems *c*. The correspondent DNA fragment, with 794 bp, was amplified by means of a PCR reaction using genomic DNA of *D. vulgaris* and the following oligonucleotides: 5′- CAA ACA TGC GCA TAT GCT TTC TGT C-3′ and 5′- GAG ACT CCT GAA AGC TTC ATG AC - 3′ with restriction sites for NdeI and HindIII, respectively. The gene was cloned in pET-28a(+) (Novagen) to allow the insertion of a sequence that encodes a 6×-His tag. The gene was then subcloned in pET22b(+) (Novagen), previously digested with NcoI and HindIII, that further allows the expression of a PelB leader sequence. The truncated subunit II of the haem–copper oxygen reductase from *D. vulgaris*, from now on referred as cytochrome *c* domain, was then produced in *E. coli* BL21-Gold (DE3) (Stratagene) harbouring a plasmid with auxiliary genes for haem *c* production (pEC86-*ccmABCDEFGH*) [22]. The cells were grown, at 37 °C, in LB medium containing 50 μg/ml of kanamycin and 20 μg/ml of chloramphenicol, until an OD_{600} ~0.7. At this point, 200 μM of IPTG (isopropyl-β-D-thiogalactopyranoside) and 50 μM of $FeSO_4$ were added to the medium. The culture was grown, at 37 °C, for extra 4 h after which the cells were collected by centrifugation.

2.2. Cloning and expression of the recombinant D. vulgaris Hildenborough cytochrome c_{553}

In the *D. vulgaris* Hildenborough genome, the cytochrome c_{553} is encoded by the *cyf* gene (DVU1817) [21], that was amplified in a PCR reaction performed with genomic DNA of *D. vulgaris* and two oligonucleotides: 5′ - GAG AAT TCC ATG AAA CGA GTT C -3′, with a EcoRI restriction site, and 5′ - TCG CTC GAG CTT GCT CAT GTA GTC - 3′, with a XhoI restriction site. The gene was then cloned directly in pET-22b(+), sequenced to confirm the absence of errors and introduced in *E. coli* BL21-Gold (DE3) cells that also contained plasmid pEC86-*ccmABCDEFGH*. To produce the recombinant protein, cells were grown in LB medium supplemented with 50 μg/ml of kanamycin and 20 μg/ml of chloramphenicol, at 37 °C, until an OD_{600} ~0.5. The expression procedure was done as above described for the production of the cytochrome *c* domain.

2.3. Protein purification

Cells overexpressing the cytochrome *c* domain or the cytochrome c_{553} were resuspended in 20 mM TrisHCl buffer, pH 7.5 (buffer A) with 20 μg/ml of DNase and disrupted in a French Press. A high speed centrifugation, at 160,000 ×*g* for 1 h, allowed the separation of the soluble fraction from the membranes, which was then loaded into a Chelating Sepharose High Performance column (GE Healthcare), previously equilibrated with $NiCl_2$ and with buffer A plus 400 mM NaCl. A linear gradient up to 400 mM imidazole was then applied and the cytochrome *c* domain protein was eluted at ~250 mM imidazole, while the cytochrome c_{553} was eluted at ~280 mM imidazole. At this stage, the cytochrome c_{553} was found to be pure, as judged by SDS-PAGE [23]. The fraction containing the cytochrome *c* domain protein was further purified in a Q-Sepharose High Performance column (GE Healthcare) to which a linear gradient up to 1 M NaCl in buffer A was applied, occurring the elution of a fraction with pure protein at ~250 mM NaCl.

2.4. Protein biochemical characterization

Protein concentration was determined by the bicinchoninic acid method [24] using protein standards from Sigma, and the hemochromopyridine method was performed according to the procedure described by Berry et al. [25].

Protein molecular mass was assessed by gel filtration in a Superdex 200 column (GE Healthcare), according to the instructions of the manufacturer and using the commercially available standards of GE Healthcare.

UV–visible spectra were recorded, at room temperature, in a Shimadzu UV-1700 spectrophotometer. A redox mixture, containing 50 mM TrisHCl pH 7.5, 2.8 μM of purified protein and 12 μM of mediators, was titrated under argon atmosphere and continuous agitation. The redox mediators used were the following: N,N-dimethyl-p-phenylene-diamine ($E'_{0,7}$=340 mV), p-benzoquinone ($E'_{0,7}$=240 mV), 1,2-naphtoquinone-4-sulfonic acid ($E'_{0,7}$=215 mV), 1,2-naphtoquinone ($E'_{0,7}$=180 mV), trimethylhydroquinone ($E'_{0,7}$=115 mV), phenazine methosulphate ($E'_{0,7}$=80 mV), 1,4-naphtoquinone ($E'_{0,7}$=60 mV), duraquinone ($E'_{0,7}$=5 mV), menadione ($E'_{0,7}$=0 mV), plumbagin ($E'_{0,7}$=−40 mV), phenazine methosulphate ($E'_{0,7}$=−125 mV), 2-hydroxy-1,4-naphtoquinone ($E'_{0,7}$=−152 mV) and anthraquinone sulfonate ($E'_{0,7}$=−225 mV). A silver/silver chloride electrode previously calibrated in a saturated quinhydrone solution at pH 7 was used. The redox titration was performed recording the entire spectra from 350–700 nm. The experimental data was analysed using MATLAB (Mathworks, South Natick, MA) for Windows, and fitted with two consecutive one-electron Nernst curves, since there was no evidence for haem–haem interactions.

The cytochrome *c* domain protein was analysed by NMR and EPR spectroscopies. For the NMR studies, the protein was prepared in D_2O and the pH and ionic strength was adjusted with 10 mM phosphate buffer, pH 6.9. Reduction of the protein was achieved by addition of small volumes of a concentrated solution of sodium dithionite. 1D ^{1}H-NMR spectra (1k scans) were acquired in a Bruker Avance 500 MHz

spectrometer using a QXI probe at 298 K. The residual water signal was saturated using a selective pulse of 500 ms. EPR spectra were recorded, at 15 K, on a Bruker EMX spectrometer, with an Oxford Instruments continuous flow helium cryostat.

2.5. Western blot analysis

The antiserum against the cytochrome *c* domain of subunit II of *D. vulgaris* oxygen reductase was produced by Centre Lago Company. To this end, 800 μg of the purified cytochrome *c* domain was applied in an SDS-PAGE gel and the protein band was cut and used for the immunization of rabbits. The serum of the rabbits final bleeding was utilized in the Western blot analysis.

D. vulgaris Hildenborough was grown anaerobically at 37 °C in sealed flasks (70 or 200 ml) in the following media: lactate/sulphate (L/S) [26]; L/S modified by substituting lactate by 40 mM pyruvate (pyruvate/sulphate-P/S); L/S where lactate was replaced by 20 mM acetate (acetate/sulphate-A/S); L/S in which lactate was replaced by formate (40 mM) and acetate (20 mM) (formate/acetate/sulphate-Fo/A/S); L/S with lactate and sulphate substituted by succinate (28 mM) and fumarate (50 mM); (succinate/fumarate-Sc/Fu); and L/S with thiosulphate used instead of sulphate (40 mM) (lactate/thiosulphate-L/T). Cells were also grown under the following stress conditions: oxygen exposure — cells grown in L/S medium at an OD_{600} of 0.6, were bubbled with O_2 gas for 3 h; iron stress — cells were grown under iron starvation conditions or iron excess by supplementation of the L/S medium with 5 μM or 60 μM of $(NH_4)_2Fe(SO_4)_2.6H_2O$, respectively; nitrate and nitrite — cells were grown in L/S medium containing 25 mM of $NaNO_3$; cells in L/S were grown until an OD_{600} of 0.5 at which point 100 mM $NaNO_3$ or 2.5 mM $NaNO_2$ was added to the culture for 5 h and 2.5 h, respectively. The nitrate and nitrite concentrations were similar to those used in the transcriptomic study of *D. vulgaris* described in [27,28]. All cells were collected by centrifugation (7000 ×*g*, 20 min), resuspended in buffer A and disrupted in a French Press. The protein content of the crude extracts was quantified by the bicinchoninic acid method and 30 μg of total protein was applied into a 12% SDS-PAGE gel. After electrophoresis the proteins were transferred to a nitrocellulose membrane (0.45 μm) (Bio-Rad) in a trans-blot semi-dry transfer cell apparatus (Bio-Rad) (1 h, 15 V). The membrane was equilibrated in dry milk 5% (w/v) dissolved in 10 mM Tris–HCl pH 7.5 with 150 mM NaCl (TBS-T buffer), followed by overnight incubation (4 °C) with the antiserum raised against the cytochrome *c* domain of subunit II of the *D. vulgaris* oxygen reductase (1:2500). The membrane was then washed with TBS-T buffer and incubated at room temperature for 1 h with the anti-rabbit IgG alkaline phosphatase conjugate (1:7500) (Sigma). After a wash of the unbound anti-rabbit IgG with TBS-T buffer, a solution containing nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Fluka) was used for detection. The Western blot was also performed using the antiserum raised against the subunit I of *Paracoccus* (*Pa.*) *denitrificans* aa_3 (1:500) (gift from Prof. Bernd Ludwig). The pre-stained STD broad range molecular marker of Bio-Rad was used in the Western blots.

2.6. Membrane preparation and oxygen reduction assays

D. vulgaris Hildenborough cells were grown anaerobically in lactate/sulphate medium, at 37 °C, in a 3-liter reactor (Applikon, Biocontroler 4DI 1030). After centrifugation, the cells were resuspended in buffer A plus 20 μg/ml DNase and disrupted. For removal of unbroken cells, the extract was centrifuged at 5000 ×*g* for 50 min and membranes were collected by centrifugation at 160,000 ×*g* for 2 h. After resuspension of the membrane fraction in buffer A, the protein content was determined. The oxygen reduction activity was determined in an Iso2 dissolved oxygen meter (World Precision Instruments, Inc). The assay was carried out at 30 °C using 2.7 mg of membranes and 0.8 mM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) as artificial electron donor, that was kept reduced with 8 mM of sodium ascorbate. To test if the cytochrome c_{553} could act as electron donor for the *D. vulgaris* membranes, in the assay TMPD was replaced by 6.3 μM of purified *D. vulgaris* cytochrome c_{553}. In all cases, inhibition of the oxygen reduction activity was achieved by the addition of 20 μM of KCN.

3. Results and discussion

3.1. Sequence analysis of D. vulgaris haem–copper oxygen reductase

In the genome of *D. vulgaris* Hildenborough, the gene cluster DVU1810–DVU1816 comprises the genes coding for a putative haem–copper oxygen reductase (Fig. 1) [21]. This cluster is formed by six genes, encoding two hypothetical proteins (DVU1810, DVU1816), a protohaem IX farnesyltransferase (DVU1811), orthologs of subunits I (DVU1815) and II (DVU1812), characteristic of this superfamily, and orthologs of subunit III (DVU1814) and subunit IV (DVU1813). The sequence of subunit I lacks the helix VI glutamyl (E278, *Pa. denitrificans* numbering) at the end of the D-channel; instead, there is a tyrosyl and a seryl in helix VI, which allows to classify this enzyme as a Type A2 haem–copper oxygen reductase. All the other residues considered important for the D- and K-proton channels are conserved. Indeed, the higher amino acid sequence similarity is found with enzymes of the A2 subfamily, ranging from 24 to 67% of identity and 39 to 83% of similarity. The residues that bind the di-copper CuA centre in oxygen reductase enzymes are also conserved in the subunit II [19]; however subunit II contains at the C-terminus two canonical motifs CxxCH for binding of two haems *c*, a situation so far not observed in any other A or B type enzymes.

The amino acid sequence of *D. vulgaris* subunit II was extensively compared with the haem–copper oxygen reductases available in the databases, and the higher similarities were found with caa_3 enzymes, namely those of subfamily A2. Dendograms performed with either subunit I or II yielded the same result, strongly suggesting that the $ccaa_3$ enzymes (so far found only in the genomes of *Desulfovibrio* species, namely in *D. vulgaris* Hildenborough, *D. vulgaris* DP4 and *D. desulfuricans*) evolved from the caa_3 oxygen reductases (Fig. 2). As observed for these latter enzymes, the cytochrome caa_3 domain bears no resemblances with the monohaem or dihaem subunits of Type C (cbb_3) oxygen reductases. A more restricted analysis was performed using only the cytochrome *c* domains of caa_3 and $ccaa_3$ enzymes. Remarkably, in the $ccaa_3$ enzymes, the two *c* domains are quite similar to each other (identities of ~44%), as well as highly similar to the single cytochrome *c* domain of the caa_3 proteins (Fig. 3). This observation strongly suggests that the dihaem domain evolved from a gene duplication event. The structure of both domains from the *D. vulgaris* enzyme were modelled, and the best template was that of the *R. marinus* caa_3 cytochrome *c* domain whose three dimensional structure revealed a novel type of cytochrome *c* fold in which there is an insertion of a small β-sheet that appears to be common to caa_3 oxygen reductases [29]. Furthermore, these models (data not shown)

Fig. 1. Gene cluster encoding the haem–copper oxygen reductase of *D. vulgaris*: DVU1810, DVU1816 encode hypothetical proteins; DVU1811 encodes a putative protohaem IX farnesyltransferase; DVU1815, DVU1812, DVU1814 and DVU1813 encode orthologs of haem–copper oxygen reductase subunits I, II, III and IV, respectively. The gene encoding cytochrome c_{553} (DVU1817) which is in the close vicinity of the cluster is also represented.

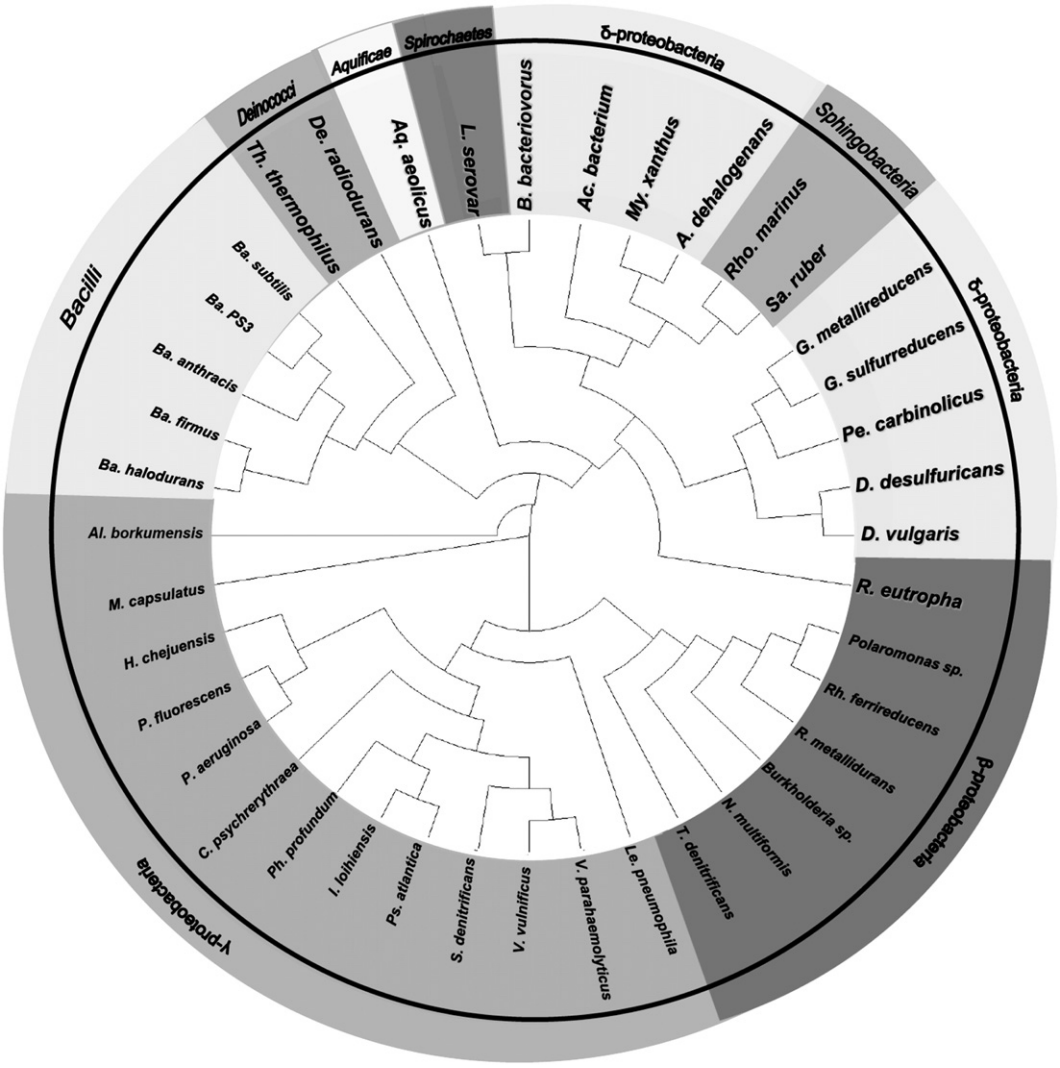

Fig. 2. Dendogram of the subunit II of caa_3 and $ccaa_3$ (*D. vulgaris* and *D. desulfuricans*) enzymes. The organism names (in bold letters) represent enzymes from the A2 subfamily. Organism and NCBI accession number: *D.* (*Desulfovibrio*) *vulgaris* (AAS96289.1), *D. desulfuricans* (ABB38623.1), *G.* (*Geobacter*) *metallireducens* (ABB30497.1), *G. sulfurreducens* (AAR33556.1), *Pe.* (*Pelobacter*) *carbinolicus* (ABA89767.1), *L.* (*Leptospira*) *serovar* (AAN47441.1), *B.* (*Bdellovibrio*) *bacteriovorus* (CAE77943.1), *Sa.* (*Salinibacter*) *ruber* (ABC44510.1), *A.* (*Anaeromyxobacter*) *dehalogenans* (ABC80578.1), *My.* (*Myxococcus*) *xanthus* (ABF90254.1), *Ac.* (*Acidobacteria*) *bacterium* (ABF41997.1), *R.* (*Ralstonia*) *eutropha* (AAZ62990.1), *Rh.* (*Rhodoferax*) *ferrireducens* (ABD69410.1), *Polaromonas sp.* (ABE43196.1), *R. metallidurans* (ABF07147.1), *Burkholderia sp.* 383 (ABB09770.1), *N.* (*Nitrosospira*) *multiformis* (ABB75074.1), *T.* (*Thiobacillus*) *denitrificans* (AAZ96278.1), *V.* (*Vibrio*) *vulnificus* (AAO07514.1), *V. parahaemolyticus* (BAC61879.1), *S.* (*Shewanella*) *denitrificans* (ABE56792.1), *Ph.* (*Photobacterium*) *profundum* (CAG18606.1), *I.* (*Idiomarina*) *loihiensis* (AAV81103.1), *Ps.* (*Pseudoalteromonas*) *atlântica* (ABG42745.1), *C.* (*Colwellia*) *psychrerythraea* (AAZ27294.1), *P.* (*Pseudomonas*) *fluorescens* (ABA71823.1), *P. aeruginosa* (AAG03495.1), *H.* (*Hahella*) *chejuensis* (ABC26969.1), *M.* (*Methylococcus*) *capsulatus* (AAU92995.1), *Le.* (*Legionella*) *pneumophila* (CAH14115.1), *Al.* (*Alcanivorax*) *borkumensis* (CAL17348.1), *Th.* (*Thermus*) *thermophilus* (AAA27484.1), *Aq.* (*Aquifex*) *aeolicus* VF5 (NP_214503.1), *De.* (*Deinococcus*) *radiodurans* R1 (NP_296338.1), *Rho.* (*Rhodothermus*) *marinus* (CAC08531.1), *Ba.* (*Bacillus*), *firmus* (AAA22364.1), *halodurans* (BAB06334.1), *sp. PS3* (BAA03045.1), *anthracis* (AAP27878.1), *subtilis* (CAB13362.1).

indicate for both haems a His-Met haem–iron axial coordination, in agreement with the amino acid sequence comparison (Fig. 3) and the NMR data (see below), with Met278 and Met378 (*D. vulgaris* numbering) strictly conserved among $ccaa_3$ enzymes and which are the most probable methionine ligands.

3.2. Characterization of the dihaem cytochrome c domain of subunit II of D. vulgaris haem–copper oxygen reductase

In order to biochemically characterize the cytochrome *c* domain of the subunit II haem copper oxygen reductase, the truncated protein

Fig. 3. Amino acid sequence alignment of the haem *c* domains of *D. vulgaris* Hildenborough and *D. desulfuricans* G20 $ccaa_3$ haem–copper oxygen reductase subunit II (*c*1 — first haem *c* domain; *c*2 — second haem *c* domain) and *Rho. marinus* DSM 4252 caa_3 haem–copper oxygen reductase subunit II (*c* — haem *c* domain). The canonical motif CxxCH for binding of haem *c* is indicated on top of the alignment and the probable methionine ligands of the haem–iron axial coordination are also indicated (*). The strictly conserved amino acid residues are shaded in black and the conserved residues are shaded in gray. The alignment was made with Clustal W2 [39].

consisting solely of the C-terminal domain (named cytochrome *c* domain) was produced, purified and analysed.

The cytochrome *c* domain protein migrated in SDS-PAGE with an apparent molecular mass of approximately 29 kDa, in accordance with the predicted mass plus the extra 6×-His tag tail. The UV–visible spectrum of the purified protein exhibits in the oxidized state a Soret band at 409 nm and a broad band between 500 and 600 nm (Fig. 4A). After reduction with sodium dithionite, the Soret band shifted to 416 nm and the β and α band appeared at 521 and 550 nm, respectively, the typical spectral profile for a reduced cytochrome *c* (Fig. 4A). The absorption maximum of the pyridine and redox pyridine hemochrome of the cytochrome *c* domain showed a band at 550 nm, confirming the presence of haem *c* (data not shown), with a ratio of 1.5±0.2 haem per protein. The redox titration performed at pH 7.5, could be described as a sum of two one-electron Nernst equations, with reduction potentials of −100 mV for the low-potential haem and +110 mV for the for the high-potential haem, with relative contributions of 0.4 and 0.6, respectively (Fig. 4B). With such a large difference of reduction potentials possible homotropic haem–haem interactions are not observable. It should be stressed that these reduction potentials may not reflect the values in the intact protein, where the cytochrome domain will be sensing a different environment. The distinct contributions for the absorption data may result from slightly absorption coefficients of the two haems, but both show identical absorption maxima in the Soret and α bands.

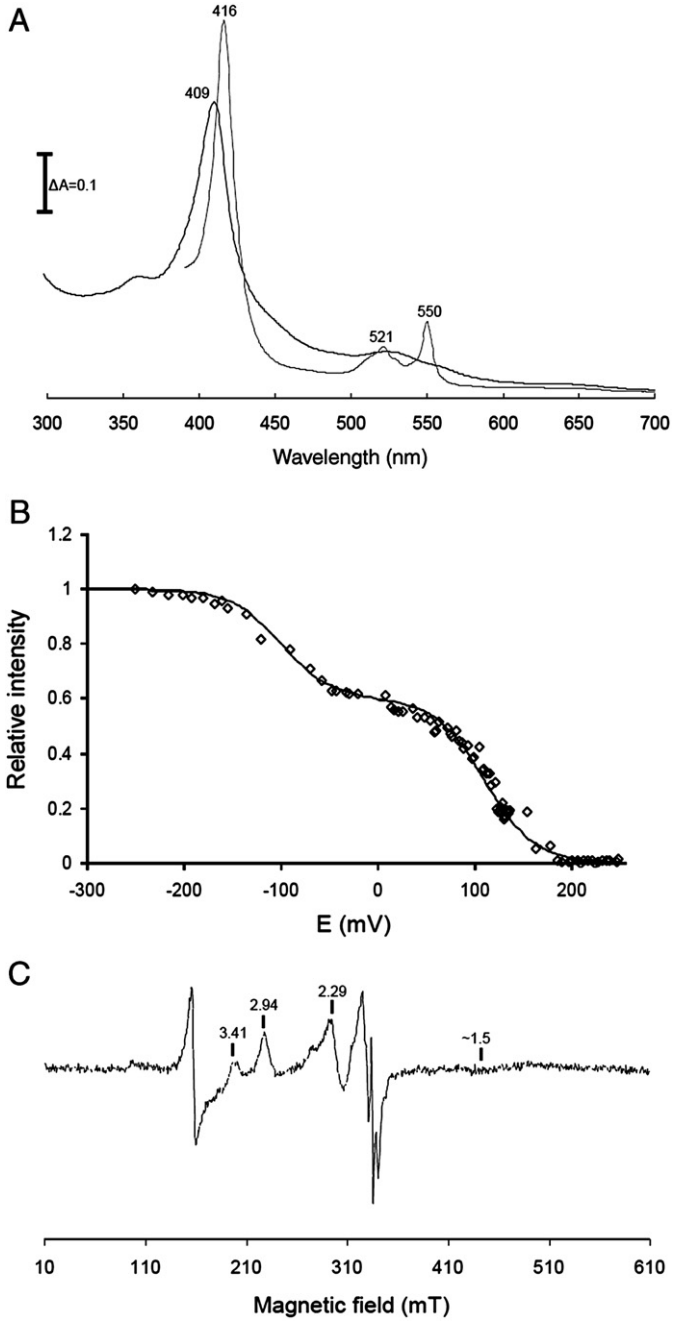

Fig. 4. Characterization of the dihaem cytochrome *c* domain of subunit II haem–copper oxygen reductase of *D. vulgaris* Hildenborough. (A) UV–visible spectrum of the oxidized (black line) and reduced (dashed grey line) form of the cytochrome *c* domain. (B) Redox titration (monitored at the Soret band), fitted to the sum of two one-electron Nernst equations, with reduction potentials of −100 mV and +110 mV for the low-potential and high-potential haem and with a contribution of 0.4 and 0.6, respectively. (C) EPR spectrum of cytochrome *c* domain, at 10 K, 9.39 GHz and 2 mW.

In order to identify the ligands of the haems *c* the protein was analysed by NMR spectroscopy. The 1D ^{1}H-NMR spectrum of the oxidized form of the cytochrome *c* domain showed features characteristic of a protein containing low-spin paramagnetic haems. After reduction of the enzyme, the paramagnetic signals in the high frequency region disappear, and the spectrum showed the fingerprint of a typical diamagnetic haem protein that has a histidine–methionine coordinated haem, i.e., it exhibits a signal in the low frequency region at −2.76 ppm that corresponds to the methionine ε methyl (data not shown) [30,31]. The EPR spectrum of the oxidized cytochrome *c* domain has *g*-values of 3.41, 2.94, 2.29, and ~1.5 confirming the presence of two haems *c* in the low-spin state (Fig. 4C). Spectral simulations indicated that the two haems are present in a ~1:1 ratio, one with principal *g*-values of 2.94, 2.29 and 1.51 (rhombic ligand field) and the other of the "strong g_{max}" type, with only the resonance at $g=3.41$ well observed (axial ligand field at the haem iron). The resonances at $g=4.3$ and $g\sim2.0$ are due to minor amounts of contaminants.

3.3. Analysis of the expression of the *D. vulgaris* haem–copper oxygen reductase

The expression of *D. vulgaris* haem–copper oxygen reductase subunit II was analysed by Western blotting of crude extracts of cells grown under different conditions and using the antibody raised against the cytochrome *c* domain of subunit II of the enzyme. In all the conditions tested, expression of subunit II of the haem–copper oxygen reductase was detected by the development of a band around 54 kDa, which corresponds to the molecular mass of the complete subunit II.

D. vulgaris cells were grown with different electron donors or acceptors and submitted to different stresses. The electron donors used were lactate, pyruvate, acetate and formate/acetate, having sulphate as electron acceptor. It was also analysed the effect of thiosulphate as electron acceptor using lactate as electron donor, and that of succinate and fumarate replacing lactate and sulphate, respectively. The results showed that the expression of the haem–copper oxygen reductase in *D. vulgaris* cells was essentially not affected by the type of electron donor or acceptor used for the anaerobic metabolism (Fig. 5).

Nitrite was previously reported to inhibit sulphate reduction by *D. vulgaris* cells even in the presence of a sub-lethal concentration of nitrite, i.e. 2.5 mM [27]. Our data showed that addition of nitrite did not alter the expression of the subunit II of the haem–copper oxygen reductase of *D. vulgaris* (Fig. 5). This result is in accordance with that obtained in transcriptional studies, where no significant difference in the gene expression level was observed upon exposure to similar conditions of nitrite stress [28]. Likewise, the inclusion of nitrate in the medium in any of the concentrations tested did not lead to a significant variation in the band intensities (Fig. 5).

Since the oxygen and iron metabolism are linked [32] the influence of the iron concentration on the expression of *D. vulgaris* haem–copper oxygen reductase was also evaluated. It was observed that an

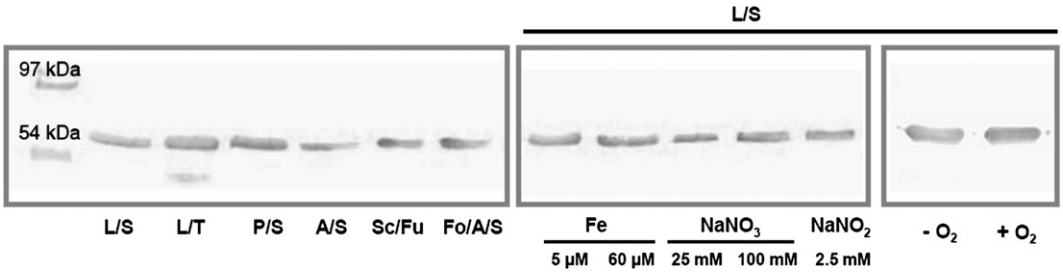

Fig. 5. Expression analysis of subunit II oxygen reductase in cells of *D. vulgaris* grown under different conditions accessed by Western blot performed with an antiserum raised against the cytochrome *c* domain. The cell extracts were applied in three independent SDS-PAGE gels: L/S (lactate/sulphate); L/T (lactate/thiosulphate) P/S (pyruvate/sulphate); A/S (acetate/sulphate); Sc/Fu (succinate/fumarate); Fo/A/S (formate/acetate/sulphate); L/S supplemented with 5 or 60 μM of iron (Fe); L/S supplemented with 25 mM of nitrate ($NaNO_3$); L/S with 100 mM of nitrate ($NaNO_3$); L/S in the presence of 2.5 mM of nitrite ($NaNO_2$); growth in L/S and exposed O_2. The 54 and 97 kDa bands of the molecular weight marker are also displayed.

excess or deficiency of iron does not affect the expression of the subunit II of the enzyme (Fig. 5).

Previous transcriptomic analysis of *D. vulgaris* showed that growth of cells in the presence of 0.1% of oxygen causes a quite small increase in the transcription of the gene encoding subunit II of the haem–copper oxygen reductase [33]. Accordingly, when we investigated the expression of the enzyme no meaningful difference was also observed between cells of *D. vulgaris* submitted to oxygen and those grown anaerobically (Fig. 5).

The expression of the *D. vulgaris* subunit I of haem–copper oxygen reductase was also analysed in cells grown under all the above-mentioned conditions, using the antibody against subunit I of the aa_3 haem–copper oxygen reductase of *Pa. denitrificans*, which cross reacts with *D. vulgaris* subunit I. The results revealed that, as observed for subunit II, the expression of subunit I is independent of the different growth conditions tested (data not shown). Hence, the expression of the haem–copper oxygen reductase seems to be constitutive.

3.4. Cytochrome c_{553} gives electrons to membranes of *D. vulgaris*

D. vulgaris membranes were found to be able to reduce oxygen when accepting electrons from the artificial electron donor TMPD, with a TMPD:oxygen oxidoreductase activity of 12±4 μmol O_2 min^{-1} mg^{-1} (Table 1). Upon addition of KCN, a well known inhibitor of the haem–copper oxygen reductase family, the rate of oxygen reduction decreased by 35%, providing evidence for the presence of an active haem–copper oxygen reductase in the membranes of *D. vulgaris*. Note that the addition of KCN up to 500 μM did not significantly change the percentage of inhibition (data not shown).

One of the possible electron carriers for the haem–copper oxygen reductase is the cytochrome c_{553}, encoded by the gene DVU1817, which is located in the vicinity of the gene cluster encoding the oxygen reductase of *D. vulgaris* (Fig. 1). This gene is also present in the vicinity of the genes for subunits of the haem–copper oxygen reductase in the genomes of *D. desulfuricans* G20, *D. vulgaris* DP4 and *D. vulgaris* Miyazaki [34]. In order to assess if the cytochrome c_{553} of *D. vulgaris* was able to give electrons for the haem–copper enzyme, TMPD was replaced by the ascorbate-reduced cytochrome c_{553} that has a midpoint potential of +62 mV [35]. The value of activity determined (5±1 μmol O_2 min^{-1} mg^{-1}) indicated that the monohaem cytochrome c_{553} may serve as electron donor to *D. vulgaris* membranes during the oxygen reduction process. In this case it was also observed that addition of KCN caused partial inhibition of oxygen consumption (Table 1).

Table 1
Oxygen reduction activities performed in the *D. vulgaris* membranes using TMPD or cytochrome c_{553} as electron donors

	Activity (μmol O_2 min^{-1} mg $protein^{-1}$)	KCN inhibition (%)
TMPD:oxygen oxidoreductase	12±4	35
Cytochrome c_{553}:oxygen oxidoreductase	5±1	22

4. Conclusion

In this work, we have shown that *D. vulgaris* Hildenborough contains a haem–copper oxygen reductase of the A2 type, which has the particularity, so far shared only with other *Desulfovibrio* species, of having a dihaem cytochrome *c* domain at the C-terminus of subunit II. The enzyme appears to be constitutive, under the various conditions tested, and is active. It was shown that the monohaem cytochrome c_{553} is able to transfer electrons to the membranes of *D. vulgaris*, enabling oxygen consumption. Although *Desulfovibrio* does not contain a quinol:cytochrome *c* oxidoreductase (bc_1 complex), an ortholog for an alternative complex III, first identified in *Rho. marinus* at the biochemical level [36] and later at the genetic level [37], is present, as proposed previously by Yanyushin et al. [38].

The actual function of oxygen reductases in sulphate reducing bacteria remains to be clarified, since so far only *D. desulfuricans* ATCC 27774 cells were reported to sustain growth in the presence of oxygen [4]. In fact, many enzymes from *Desulfovibrio* species, involved in sulphur and hydrogen metabolisms, among others, are extremely oxygen sensitive and proven to be reversibly or irreversibly inactivated by O_2, or even completely damaged by oxygen. Nevertheless, it was not yet proved if the main function of these enzymes is to contribute to energy conservation or if they are dedicated to fulfil a major role in oxygen detoxification, protecting the anaerobic metabolism of these bacteria against traces of oxygen present in the neighbouring environment and, at the same time, enabling energy conservation by oxidative phosphorylation.

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